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Review

The biology of the opioid growth factor receptor (OGFr)

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Abstract

Opioid peptides act as growth factors in neural and non-neural cells and tissues, in addition to serving for neurotransmission/neuromodulation in the nervous system. The native opioid growth factor (OGF), [Met⁵]-enkephalin, is a tonic inhibitory peptide that plays a role in cell proliferation and tissue organization during development, cancer, cellular renewal, wound healing, and angiogenesis. OGF action is mediated by a receptor mechanism. Assays with radiolabeled OGF have detected specific and saturable binding, with a one-site model of kinetics. Subcellular fractionation studies show that the receptor for OGF (OGFr) is an integral membrane protein associated with the nucleus. Using antibodies generated to a binding fragment of OGFr, this receptor has been cloned and sequenced in human, rat, and mouse. OGFr is distinguished by containing a series of imperfect repeats. The molecular and protein structure of OGFr have no resemblance to that of classical opioid receptors, and have no significant homologies to known domains or functional motifs with the exception of a bipartite nuclear localization signal. Immunoelectron microscopy and immunocytochemistry investigations, including co-localization studies, have detected OGFr on the outer nuclear envelope where it interfaces with OGF. The peptide–receptor complex associates with karyopherin, translocates through the nuclear pore, and can be observed in the inner nuclear matrix and at the periphery of heterochromatin of the nucleus. Signal transduction for modulation of DNA activity is dependent on the presence of an appropriate confirmation of peptide and receptor. This report reviews the history of OGF–OGFr, examines emerging insights into the mechanisms of action of opioid peptide–receptor interfacing, and discusses the clinical significance of these observations. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Endogenous opioid peptides, discovered by Hughes and coworkers [21] in 1975 are known to be potent regulators of growth [107–111,116,129,132,148], as well as neuro-modulators/neurotransmitters [1]. One native opioid peptide, [Met⁵]-enkephalin, encoded by the preproenkephalin A (PPE) gene, has been identified as a negative growth regulator [123,128,129,132,148]. To distinguish the role of [Met⁵]-enkephalin as a growth factor in neural and non-neural cells and tissues, and in prokaryotes and eukaryotes, this peptide was termed opioid growth factor (OGF). OGF is broad-based in action and functions in development, cellular renewal, cancer, wound healing, and angiogenesis [4,5,9,11,15,18,26,29,32,35,38,42,43,45,47,49,53,57,59,67,70,71,73–75,84,86,98–101,119,123,128,130,134,135,137–139,141,142,144,145,147,154–158]; whether related compounds such as [D-Met², Pro⁵]-enkephalinamide are related to OGF activity (and the OGF receptor) is unclear [33,34,60,81–83].

The biological effects of OGF can be blocked by naloxone and are stereospecific, pharmacological characteristics suggesting involvement of an opioid receptor [39,63]. Previous studies have reported that in neural tissues [Met⁵]-enkephalin-OGF—has considerable selectivity for the δ opioid receptor and, to a slightly lesser extent, the μ opioid receptor [39]. Therefore, it was not unreasonable to conjecture that OGF action on growth processes was transduced by one or both of these opioid receptors. However, the function (growth), tissue distribution (neural and non-neural), subcellular location (nuclear-associated), ligand specificity (i.e. [Met⁵]-enkephalin), and competitive inhibition profile of OGF differed appreciably from what is known about the characteristics of classical opioid receptors [39]. Based on the fulfillment of the pharmacological principles of an opioid receptor, the receptor for OGF was originally named the zeta (ζ) opioid receptor [93,95,96]. Molecular studies [150–152] now show that the genomic and proteomic nature of this receptor is distinctly different from classical opioid receptors, and the receptor has been renamed Opioid Growth Factor receptor (OGFr). This review is intended to summa-

size our knowledge of the cell and molecular biology of the OGF receptor.

2. Identification of the opioid growth factor

The early literature on the discovery of the OGF receptor has been thoroughly reviewed elsewhere [132]. To bring continuity to this chapter about the cellular and molecular biology of the OGF receptor, however, a brief summary is presented.

The exciting hypothesis that endogenous opioid peptides function in growth regulation of normal and abnormal cells and tissues put forth in the early 1980s [89,102–111], was postulated on the basis of a number of crucial experimental observations. Some of these included: (i) alterations in cell/tissue/body growth and DNA synthesis *in vitro* and *in vivo* by opioid antagonists or a synthetic enkephalin analog [3,23,52,83,89,102–111,126,143,153], (ii) the effects of opioid antagonists on growth were related to the duration of opioid receptor blockade rather than drug dosage [111,125], (iii) an accelerated growth resulting from the absence of opioid-receptor interfacing, thereby inferring that endogenous opioids are inhibitory molecules [107–111], (iv) deceleration in growth by intermittent opioid receptor blockade, suggesting that compensating high levels of opioids interact with elevated levels of receptors to cause a supersensitive growth inhibition at the cessation of opioid-receptor antagonism [16,17,23,52,106–115,117,119,120,122–124,126,143,153], (v) the stereospecific nature of endogenous opioid activity on growth response [112,122], (vi) presence of endogenous opioid peptides in developing cells and tissues [58,79,80,85,137], (vii) detection of opioid receptors in the developing nervous system [7,8,77–80], (viii) transient expression of receptor binding to radiolabeled [Met⁵]-enkephalin during cerebellar development, with maximal binding occurring in the first week of life and declining thereafter [77–80], and (ix) discovery of opioid receptors in the somatic tissues of developing organisms [14]. Contributing to the formulation of this hypothesis was recognition of reports that exogenous opioids such as methadone and morphine altered the

pattern of growth both in vitro and in vivo [131], although it was acknowledged that the mechanisms of action leading to changes in growth could be different between exogenous and endogenous opioids.

A critical issue at this juncture concerned the need to identify the endogenous opioid peptide(s) involved with growth. Early experiments had indicated that [Met⁵]-enkephalin acted as an antitumor agent with murine B16 melanoma [57,71], L1210 leukemia [65], and S20Y neuroblastoma [121]; however, a closely related compound, [Leu⁵]-enkephalin, had no inhibitory effect [121]. It was also known that β -funaltrexamine (β -FNA), a highly selective and irreversible μ opioid receptor antagonist [136], as well as the δ and κ opioid receptor selective compounds [D-Ala², D-Leu⁵]-enkephalin (DADLE) and ethylketocyclazocine (EKC), respectively, did not influence tumor incidence or host survival in rodents with transplanted murine neuroblastoma [136]. In addition, β -FNA had no effect on the regulation of body and brain development in rats [118]. Studies on DNA synthesis and endogenous opioid peptides, showed that [Met⁵]-enkephalin had a marked effect on the labeling index of the external germinal (granule) cells in the cerebellum of 6-day-old rats [119,128]. The proliferation of the germinative cells in the developing brain was not altered by concomitant treatment with [Met⁵]-enkephalin and the

short acting, low potency opioid antagonist—naloxone, or by naloxone alone, indicating opioid receptor mediation of function. These results supported the hypothesis that endogenous opioid peptides participated in growth, but raised the need for a systematic investigation of opioid peptides as growth modulators.

Using murine neuroblastoma cells (both in vivo and tumor transplantation) [121,123], and in subsequent studies with the developing rat cerebellum [128], [Met⁵]-enkephalin (OGF) was discovered to be the most potent opioid peptide associated with growth (Table 1). In the case of tumor cells, peptide concentrations as low as 10^{-10} M inhibited the growth of log-phase S20Y neuroblastoma cells exposed to drug for 48 h, and heptapeptide [Met⁵,Arg⁶,Phe⁷]-enkephalin, octapeptide/proenkephalin [Met⁵,Arg⁶,Gly⁷,Leu⁸]-enkephalin, and [Leu⁵]-enkephalin also exhibited inhibitory properties to some extent; all of these peptides are derivatives of the PPE family. Experiments employing analogues of [Met⁵]-enkephalin revealed the selectivity of this peptide. For example, no changes in cell growth occurred when the amino acids Tyr¹ or Met⁵ were deleted, when smaller fragments of the [Met⁵]-enkephalin molecule such as Tyr-Gly, Tyr-Gly-Gly, or even Met alone were used, or when alterations in the basic structure of [Met⁵]-enkephalin (e.g. [Met⁵]-enkephalinamide, [Met⁵]-enkephalin sulfoxide) were made.

Table 1

Effect of opioid peptides and analogs related to proenkephalin A, prodynorphin, and proopioidmelanocortin, on the number of murine S20Y neuroblastoma cells in tissue culture after 48 h of drug exposure

Compound	Structure	Peptide concentration (M)		
		10^{-6}	10^{-8}	10^{-10}
L-Tyrosylglycine	Tyr-Gly			
Tyr-Gly-Gly	Tyr-Gly-Gly			
[Des-Met ⁵]-enkephalin	Tyr-Gly-Gly-Phe			
[Des-Tyr ¹ -Met ⁵]-enkephalin/ β -lipotropin ₆₂₋₆₅	Gly-Gly-Phe-Met			
[Met ⁵]-enkephalin	Tyr-Gly-Gly-Phe-Met	47**	59**	62**
[Met ⁵]-enkephalinamide	Tyr-Gly-Gly-Phe-Met-NH ₂			
[Met ⁵ (O)]-enkephalin (sulfoxide)	Tyr-Gly-Gly-Phe-Met-(O)			
[Met ⁵ ,Lys ⁶]-enkephalin	Tyr-Gly-Gly-Phe-Met-Lys			
[Met ⁵ ,Arg ⁶ ,Phe ⁷]-enkephalin/heptapeptide	Tyr-Gly-Gly-Phe-Met-Arg-Phe	71**	77**	69**
[Met ⁵ ,Arg ⁶ ,Gly ⁷ ,Leu ⁸]-enkephalin/octapeptide/proenkephalin	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu	70**	76**	
Metorphinamide/adrenorphin	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH ₂			
BAM-12P (bovine adrenal medulla dodecapeptide)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Arg-Pro-Glu			
β -Endorphin/ β -lipotropin ₆₁₋₆₄	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu			
Peptide F	Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met			
[Leu ⁵]-enkephalin	Tyr-Gly-Gly-Phe-Leu	62**	71**	
Dynorphin A ₁₋₆	Tyr-Gly-Gly-Phe-Leu-Arg			
Dynorphin A ₁₋₇	Tyr-Gly-Gly-Phe-Leu-Arg-Arg			
Dynorphin A ₁₋₁₃	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys			
α -Neo-endorphin ₁₋₆	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr			
Dynorphin B/rimorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr			

Values represent mean cell number as percentage of control, only when statistically significant differences were recorded.

**Significantly different from controls at $P < 0.01$. Modified from data presented in Zagon and McLaughlin [123].

Other opioid peptides and analogs related to the prodynorphin and pro-opiomelanocortin genes (e.g. β -endorphin, α -neo-endorphin, dynorphin B/rimorphin), compounds selective for μ , δ , or κ opioid receptors (e.g. β -FNA, DADLE, EKC), and an extensive list of opioid (e.g. methadone, 1- α -acetylmethadol) and non-opioid (e.g. somatostatin) drugs had no significant influence on cultured cells even at concentrations as great as 10^{-6} M. The question may be raised whether the native opioid peptide for modulating the growth of tumor cells was a singular opioid compound, or whether different native opioid peptides were responsible for different tissues and/or conditions (e.g. wound healing). In a wide variety of cells and tissues examined (e.g. colon cancer, heart, squamous cell carcinoma of the head and neck, pancreatic cancer, developing nervous system) in humans and animals, both in vitro and in vivo, [Met⁵]-enkephalin has been identified as the primary opioid peptide involved with growth [50,53,98,121,123,128,147]; any other opioid peptides altering growth were related to other products of the PPE gene encoding OGF.

In subsequent experiments with tumor cells, tissues undergoing cellular renewal, developing neural and non-neural cells and tissues, angiogenesis, and wound healing, other characteristics of the OGF system were revealed [4,5,20,22,24–27,47–49,53,66,86,87,90,91,98–101,119,123,127,128,130,139,141,142,144–147,154–159]. OGF had a potent, reversible, and species and tissue non-specific action on growth. This neuropeptide was found to be autocrine and possibly paracrine produced, secreted, and effective at physiological concentrations. OGF had a rapid, direct, and stereospecific biological action, did not induce necrosis or programmed cell death (unpublished observations), functions under anchorage-dependent and anchorage-independent conditions (unpublished observations), was obedient to the intrinsic rhythms of the cell (e.g. circadian rhythm), and can pass through the placenta to the embryo/fetus. The peptide is targeted to cell proliferation (delaying the G₀/G₁ phase of the cell cycle), but also could influence cell migration, differentiation, survival, and tissue organization. Interference with OGF activity (e.g. neutralization with antibodies or application of opioid antagonists) accelerated growth by removing inhibitory signaling.

A number of points need to be emphasized on OGF action. First, this peptide, OGF, is a natural product that interacts with a native receptor to regulate growth. OGF is neither a synthetic peptide, a cytotoxic agent, nor a chemotherapeutic substance. Second, OGF functions in concert with the body's own machinery in controlling biological processes. It can be manipulated to have an exaggerated function, as in the case of adding agonist (OGF), or an absence of function such as the deprivation of OGF with opioid receptor blockade. However, this modulation of OGF activity is maintained within the boundaries of natural growth processes. Third, considera-

tion of the full spectrum of OGF activity has to take into account the changes occurring with the addition of OGF, and the elimination of OGF (e.g. opioid receptor blockade, neutralization with antibodies to OGF). For example, a human pancreatic cancer cell line (PANC-1) exposed to OGF for only 24 h in a tissue culture experiment, had 32% fewer cells than controls [147]. PANC-1 cells incubated with naltrexone (NTX) had 54% more cells than control cultures. The total biological effect of OGF is a change of 86% in growth—the summation of the effects of adding exogenous OGF and removing endogenous OGF (as revealed by blockade of OGF with its receptor).

3. Identification of the receptor for OGF

With knowledge that the endogenous opioid peptide—OGF—was associated with growth, questions about the receptor mediating the action of OGF could be addressed. It was interesting that earlier experimentation has concluded that ligands selective for known opioid receptors such as μ , δ , and κ did not alter growth of cells either in vivo or in vitro. Thus, although [Met⁵]-enkephalin is known to interact with classical opioid receptors, particularly μ and δ receptors, and one might postulate that one or more of these receptors mediated peptide activity, it was curious why this peptide, but not more specific ligands that recognized these receptors, could influence growth. Therefore, rather than using binding reactions with ligands selective for known opioid receptors, binding studies with radiolabeled [Met⁵]-enkephalin, were performed. Our initial strategy was to test whether radiolabeled [Met⁵]-enkephalin exhibited binding activity to a cellular homogenate of a murine neuroblastoma cell line—S20Y—a cell line that had been extensively characterized to have opioid peptide modulation of growth. Experiments with both S20Y tissue from tumor transplantation [95] and cell culture [96] were employed. In cells maintained in tissue culture [96], for example, the results from multiple assays performed independently showed specific and saturable binding of this peptide in whole homogenates that was consistent with a single binding site (Fig. 1). Scatchard analysis revealed high affinity binding (K_d) of 1.6 nM, and a binding capacity (B_{max}) of 48.1 fmol/mg protein. Binding was dependent on protein concentration, time, temperature, and pH, and was sensitive to 100 nM, but not 5 nM, Na⁺, Ca²⁺, and Mg²⁺, and was not markedly reduced by addition of guanyl nucleotides (GppNHp at concentrations up to 500 nM). Optimal binding required protease inhibitors, and pretreatment of the tumor cell homogenates with trypsin markedly reduced [³H][Met⁵]-enkephalin binding, suggesting that the binding site was proteinaceous in character. Displacement experiments indicated that [Met⁵]-enkephalin was the most potent displacer of [³H][Met⁵]-enkephalin, although [Met⁵, Arg⁶, Gly⁷, Leu⁸]-enkephalin and [D-Ala², D-Leu⁴]-enkephalin were the

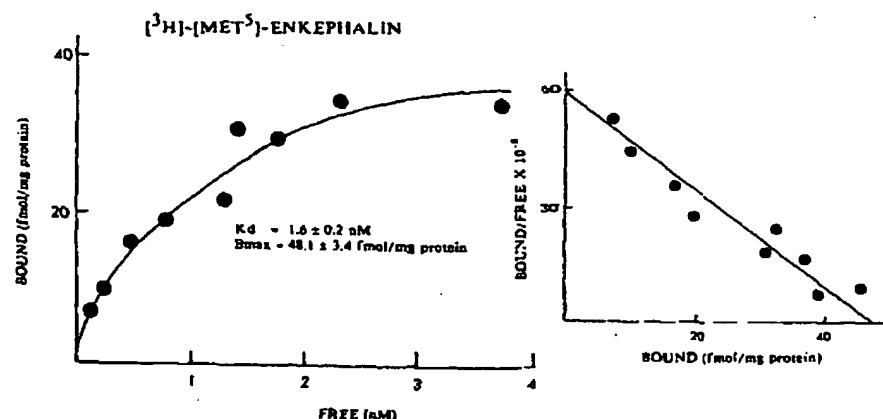


Fig. 1. Representative saturation binding isotherm and Scatchard plot (inset) of the specific binding of [^3H]-[Met 5]-enkephalin to S20Y murine neuroblastoma cell homogenates. Mean \pm S.E.M. values of binding affinity (K_d) and maximal binding capacity (B_{max}) represent multiple assays performed in duplicate (from Zagon et al. [96], with permission).

only compounds that had a K_i close (within approximately threefold) to [Met 5]-enkephalin in these whole cell homogenate preparations. Drug displacement studies revealed a requirement of an N-terminus tyrosine group as well as the greater flexibility of the C-terminus. Moreover, binding to radiolabeled [Met 5]-enkephalin displayed stereospecificity, with (–)-naloxone being 18-fold more potent than (+)-naloxone. Finally, cell density of these cancer cells did not appear to affect either binding affinity or binding capacity, with cells in log, confluent, or postconfluent cultures exhibiting similar binding values.

Experiments in the developing rat cerebellum [93], where OGF was found to be the opioid selective for modulating DNA synthesis, provided further information about the receptor for OGF. Using radiolabeled [Met 5]-enkephalin and whole homogenates of the 6-day-old rat cerebellum, a high affinity (2.2 nM) binding site with a binding capacity of 22.3 fmol/mg protein was detected. The characteristics of this binding to [Met 5]-enkephalin resembled those observed for the S20Y neuroblastoma cells. However, we gained more knowledge about OGF and discovered that the binding site was an integral membrane protein, and was associated with the nuclear fraction. Binding to [Met 5]-enkephalin in the 6-day-old rat cerebellum was maximal in the P1 (nuclear) fraction (24 \pm 3 fmol/mg protein), and no binding in the cytoplasmic fraction (P2) was recorded. In the adult cerebellum, subcellular fractionation studies assays with [Met 5]-enkephalin only showed non-opioid receptor binding, and this was related to the 39 000 \times g (P2) fraction (consistent with a plasma membrane fraction). To test our pharmacological assays, and to eliminate the possibility of erroneous results from improper subcellular fractionation procedures, assessment of μ receptors using radiolabeled [D-Ala 2 , MePhe 4 , Glyol 5]enkephalin (DAMGO) were included. In both 6-day and adult cerebellum, [^3H]DAMGO binding only was detected in the P2 fraction (24 \pm 1 and

6 \pm 1 fmol/mg protein, respectively). Similar studies were not performed with the δ receptor ligands, since no binding of radiolabeled [D-Pen 2,5]enkephalin (DPDPE) was recorded in homogenates of the 6-day-old rat cerebellum.

Thus, if indeed [Met 5]-enkephalin is known to bind to opioid receptors [39] and uses an opioid receptor to mediate its activity with respect to growth, the data emerging from functional and binding studies did not support the thesis that OGF binds to known opioid receptors. Looking at the previous literature relating [Met 5]-enkephalin to classical opioid receptors, experiments in guinea pig brain membranes, for example, indicated that [Met 5]-enkephalin had a K_i of 9.5 nM for the μ receptor, a K_i of 0.9 nM for the δ receptor, and a K_i of 4440 nM for the κ receptor [39]. Therefore, one may have suspected initially that μ and/or the δ opioid receptors, and perhaps even κ receptors, may have been mediating the action of [Met 5]-enkephalin insofar as growth. However, a number of factors were not persuasive that any of these receptors were involved with [Met 5]-enkephalin activity with regard to growth: (i) In the developing cerebellum, binding to [^3H]DAMGO and [^3H]U69,593, but not to [^3H]DPDPE, was detected, suggesting that μ and/or κ receptors but not δ receptors were targets for [Met 5]-enkephalin. However, as mentioned earlier, [^3H] [Met 5]-enkephalin binding was confined to the P1 fraction and was not associated with the P2 fraction in developing tissues, whereas [^3H]DAMGO was located solely in the P2 fraction and had no specific and saturable binding in the P1 fraction of 6-day-old rat cerebella. Furthermore, addition of a cocktail of 10 nM DAMGO, DPDPE, and U69,593 did not alter [^3H] [Met 5]-enkephalin binding in the P1 preparations, suggesting that the binding site for enkephalin was not quenched by ligands selective for classical opioid receptors such as μ , δ , or κ . (ii) In mouse S20Y neuroblastoma transplanted into A/Jax mice, where [Met 5]-enkephalin is known to alter tumor incidence and

Table 2
Binding of HT-29 nuclear (P_1) and membrane (P_2) homogenates to radiolabeled ligands selective for μ -, δ -, and κ -opioid receptors

Ligand	Receptor	Fraction	K_d (nM)	B_{max} (fmol/mg protein)
[3 H]DAMGO	μ	P_1	*	*
		P_2	13 ± 4	21 ± 5
[3 H]DPDPE	δ	P_1	*	*
		P_2	3 ± 1	10 ± 5
[3 H]U-69593	κ	P_1	*	*
		P_2	2 ± 0	14 ± 2

Data represent mean \pm S.E.M. for two independent assays.

*No specific or saturable binding (adapted from Hytek et al. [24], with permission).

growth, δ and κ , but not μ , receptors were detected. Thus, taken together with the developing cerebellum data, no one classical opioid receptor— μ or δ —was consistently present that could account for the mediation of [Met 5]-enkephalin activity. Moreover, displacement experiments of [Met 5]-enkephalin using [3 H]DADLE and [3 H]EKC in mouse neuroblastoma tissues, showed a K_i value for each radiolabeled ligand that was 800-fold or greater, suggesting that [Met 5]-enkephalin was not a selective target for either δ or κ receptors in this tissue. (iii) Rather similar observations about the specific nature of [Met 5]-enkephalin binding, and its lack of association with classical opioid receptors, as discussed in (i) and (ii) were reported in human colon cancer cells [24], human pancreatic adeno-

carcinoma [149], human head and neck squamous carcinoma [50], and rat heart [51]. For example, tissue derived from transplantation of HT-29 human cancer cells into nude mice, Hytek and colleagues [24] reported specific and saturable binding to [3 H]DAMGO, [3 H]DPDPE, and [3 H]U-69593 binding was only in the nuclear fraction (P_1) ($K_d = 6.6 \pm 0.2$ nM, $B_{max} = 256.0 \pm 12.6$ fmol/mg protein), but not in the membrane (P_2), microsomal (P_3), or soluble (S_3) fractions. In addition, these investigators showed that [3 H]DAMGO, [3 H]DPDPE, and [3 H]U-69593 binding was only in the nuclear fraction (Table 2). Displacement experiments with [3 H]Met 5 -enkephalin and nuclear homogenates of HT-29 revealed that the K_i -values of DAMGO, DPDPE, and U-69,593 were 2.6-, 5.7-, and at least 10 000-fold, greater, respectively, than [Met 5]-enkephalin, suggesting the special nature of the binding of [Met 5]-enkephalin and its lack of relationship to classical opioid receptors (Table 3). (iv) [Met 5]-enkephalin, but not ligands selective for μ , δ , and κ receptors, altered DNA synthesis and/or growth in developing rat brain, neonatal rat heart, murine neuroblastoma, human colon cancer, human neuroblastoma, human pancreatic cancer, and human head and neck squamous cell carcinoma. (v) There is a distinct developmental profile of binding to radiolabeled [Met 5]-enkephalin in human and rat brain, with maximal binding occurring at the time of cell proliferation and differentiation [92,94].

4. The OGF receptor is a receptor

A question that needs to be addressed about OGF binding is whether the element that mediates OGF is indeed a receptor. The OGF receptor was initially established on the basis of binding reactions of neural and non-neural tissues—including animal and human cancers—to radiolabeled [Met 5]-enkephalin. As stated by Leslie [39], "The major problem of interpretation in any binding study is identification of the binding site as a receptor. The key property of a binding site which allows definition as a receptor is an association with function", and "A minimum requirement for identification of a radioligand binding site as a receptor is that it is saturable, i.e. that binding can be displaced by increasing concentrations of radioactive ligand." [39]. Kenakin and colleagues [30], in discussing the definition of a receptor, state that "the main criteria for qualification for the operational term receptor are the functions of *recognition* and *transduction*. Recognition is defined by these authors as ligand selectivity, binding in a saturable manner, stereoselectivity, competitive displacement, and a presence of endogenous agonist, whereas transduction is defined as the selective interaction of receptor with a unique membrane or cytosolic component of a cell that then carries the message imparted by the drug." Kenakin et al. [30] continue that "a receptor must recognize a distinct chemical entity and translate information from that entity into a

Table 3

Potency of opioid and nonopioid ligands to compete for binding of [3 H]Met 5 -enkephalin (4.0 nM) in nuclear homogenates of HT-29 human colon tumors

Ligand	IC $_{50}$ (nM)	K_i (nM)
[Met 5]-enkephalin	16.5 ± 1.5	12.7 ± 1.2
[Met 5]-enkephalin-Arg 6 -Gly 7 -Leu 8	37.5 ± 7.5	28.9 ± 5.8
DAMGO	42.5 ± 0.5	32.7 ± 0.4
Morphine sulfate	45.0 ± 3.0	34.5 ± 2.5
β -Endorphin	71.0 ± 6.0	54.6 ± 4.6
[Leu 5]-enkephalin	75.0 ± 5.0	57.7 ± 3.9
Gastrin	77.5 ± 7.5	59.6 ± 5.8
Naltrexone hydrochloride	90.5 ± 3.5	69.6 ± 2.7
DPDPE	95.0 ± 5.0	73.1 ± 3.9
CCK-8	103 ± 2	79.0 ± 2.0
(-)-Naloxone hydrochloride	115 ± 5	88.5 ± 3.9
Dynorphin A $_{1-4}$	375 ± 25	289 ± 20
SKF-10047	795 ± 5	612 ± 4
L365260	1250 ± 250	960 ± 190
(+)-Naloxone hydrochloride	$> 10^{-5}$ M	$> 10^{-5}$ M
Somatostatin	$> 10^{-5}$ M	$> 10^{-5}$ M
L-364718	$> 10^{-5}$ M	$> 10^{-5}$ M
U-69593	$> 10^{-4}$ M	$> 10^{-4}$ M
EKC	$> 10^{-3}$ M	$> 10^{-3}$ M

Data represent means \pm S.E.M. from at least two independent assays. Equilibrium dissociation constant for [Met 5]-enkephalin was 15.4 ± 2.0 nM. IC $_{50}$ concentration inhibits 50% of maximal response; K_i , inhibitory constant; DAMGO, [D-Ala 2 , N-Me-Phe 4 , Gly 5 -ol]-enkephalin; DPDPE, [D-Pen 2 , Pen 3]-enkephalin; CCK-8, cholecystokinin octapeptide; EKC, ethylketocyclo-clazocine (adapted from Hytek et al. [24], with permission).

form that the cell can read to alter its state accordingly, e.g. by a change in membrane permeability, activation of a guanine nucleotide regulatory protein, an alteration in the transcription of DNA." "A 'completely' defined receptor would possess a unique pharmacological profile based on agonist and antagonist data, a known endogenous ligand, and a distinct amino acid sequence." Evidence assembled from the series of investigations performed in regard to OGR satisfies the definition of a receptor. Binding assays have exhibited ligand selectivity, saturability, stereospecificity, competitive displacement, and the presence of an endogenous agonist. Furthermore, OGR is an integral membrane protein that is associated with the nucleus and, after binding to OGF, functions to set off a signaling cascade related to DNA synthesis and growth.

Thus, keeping in mind that the pharmacological principles for an opioid receptor were satisfied (e.g. naloxone reversibility), our conclusion at this point was that [Met⁵]-enkephalin activity was mediated by an opioid receptor. However, in view of the function (growth), distribution (neural and non-neural cells and tissues), transient appearance during ontogeny, ligand specificity ([Met⁵]-enkephalin), competitive inhibition profile, subcellular location (i.e. nucleus), and the fact that ligands for other opioid receptors do not influence *in vivo* or *in vitro* growth, we reached the conclusion that this was an opioid receptor that possessed such a unique set of properties it warranted distinction from other opioid receptors. Hence, since this receptor was related to proliferation of cells and tissues, and the Greek word for life is 'zoe', this new opioid receptor was termed zeta (ζ), the sixth letter of the Greek alphabet [93,95,96].

5. Identification of binding fragments of the OGF receptor and generation of antibodies

Although a great deal was known about the pharmacology, biochemistry, and function of the OGF receptor, little information was available about its structure. To this end, a ligand blotting technique was employed. The method of ligand blotting is dependent on the renaturation of the polypeptides related to a protein. Utilizing one-dimensional gel electrophoresis and 1.5 nM [¹²⁵I][Met⁵]-enkephalin, four bands: 32, 30, 17, and 16 kDa were detected on autoradiograms of whole homogenates of the 6-day-old rat cerebellum (Fig. 2). These bands were not present when excess cold [Met⁵]-enkephalin (10⁻⁶ M) was added to the incubation mixture, nor was a reaction product visible when 10⁻⁶ M (–)-naloxone or naltrexone were included with [¹²⁵I][Met⁵]-enkephalin. However, bands of reaction were visible when 10⁻⁶ M (+)-naloxone was added to the radiolabeled ligand solution, showing stereospecificity of ligand binding under these conditions. The ligand blotting was dependent on time. No binding

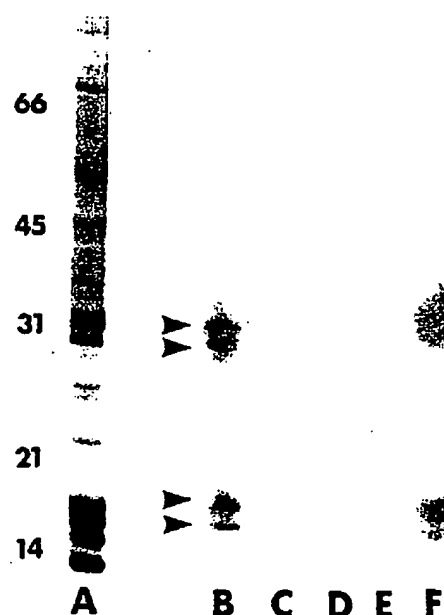


Fig. 2. Cerebellar homogenates from 6-day-old (A–C, E, F) and adult (D) rats separated on SDS–PAGE (80 μ g protein/lane). Tissues were prepared according to earlier reports [93] and a P0 fraction obtained by centrifugation at 39 000 \times g in a solution of Tris buffer and protease inhibitors. (A) Coomassie blue-stained gel showing proteins. (B–F): SDS–PAGE gels electroblotted to nitrocellulose and incubated with 1.5 nM [¹²⁵I][Met⁵]-enkephalin (B,D) plus 10⁻⁶ M [Met⁵]-enkephalin (C), 10⁻⁶ M (–)-naloxone (E), or 10⁻⁶ M (+)-naloxone (F). Note that binding fragments of 32, 30, 17, and 16 kDa are revealed in (B) and (F). binding was blocked by an excess of cold peptide (C) or by the (–) isomer of an opioid antagonist (E), and was stereospecific (compare E and F). In contrast to the 6-day-old cerebellum (B, F), no binding was recorded in adult cerebellum (D). Molecular weight ($\times 10^{-3}$ Da) markers are indicated. Arrowheads show 32-, 30-, 17-, and 16-kDa polypeptides (from Zagon et al. [97], with permission).

was visible when homogenates of adult rat cerebellum were used, indicating temporal dependence.

Subcellular fractionation of homogenates of 6-day-old rat cerebellum showed that the four binding fragments were present in autoradiograms of the nuclear fraction (P1). Little or no binding to [¹²⁵I][Met⁵]-enkephalin could be detected in P2, P3, or S3 fractions (Fig. 3). Receptor-binding assays of the four fractions were performed and, as noted in earlier experiments, specific and saturable binding was only recorded in the P1 fraction with a K_d of 2.1 ± 0.6 nM and a B_{max} of 27.0 ± 4.5 fmol/protein. In a need to further refine the isolation of these bands of binding reactivity to basic proteins, ligand blotting of electroblotted proteins prepared with non-equilibrium gel electrophoresis (NEPHGE) and SDS–PAGE was investigated. Four spots of reactivity to [¹²⁵I][Met⁵]-enkephalin were detected (Fig. 4). Therefore, a ligand blotting technique could be used to identify receptor binding polypeptides, with evidence that the renatured proteins were associated with the OGF

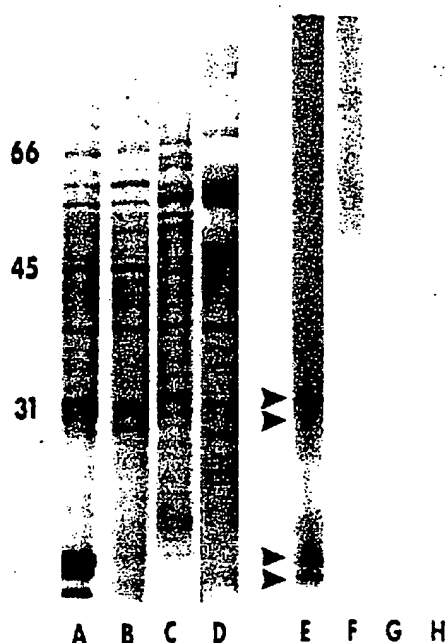


Fig. 3. Subcellular fractionation of 6-day-old rat cerebellum through a sucrose density gradient, prepared for SDS-PAGE and stained with Coomassie blue (A–D) or electroblotted and incubated in [125 I][Met 5]-enkephalin (E–H). Lanes A, E: nuclear fraction (P1) (2200 \times g); lanes B, F: membrane fraction (P2) (39 000 \times g); lanes C, G: microsomal fraction (P3) (100 000 \times g pellet); and lanes D, H: soluble fraction (S3) (100 000 \times g supernatant). Binding of [125 I][Met 5]-enkephalin to polypeptides of 32, 30, 17, and 16 kDa (arrowheads) can be observed in the autoradiograms of the P1 (E). Some faint bands of radioactivity corresponding in molecular weight to those in P1 could sometimes be detected in preparations of P3 (G). Molecular weight ($\times 10^3$ Da) markers are indicated. Each lane contained 80 μ g protein (from Zagon et al. [97], with permission).

receptor. First, the K_d of the receptor in the developing cerebellum was in the range of 2–3 nM as recorded in binding assays, and this peptide concentration was known to have a profound influence on DNA synthesis [123]. The concentration of radiolabeled [Met 5]-enkephalin used in the ligand blotting assays was 1.5 nM, indicating that the interaction of the renatured polypeptides with the ligand was physiologically relevant. Second, the ligand blotting reaction was consonant with at least two hallmarks of opioid receptors: displacement by opioid antagonists and stereospecificity [39,63]. The opioid antagonists naloxone and naltrexone eliminated binding of the ligand, however only the (–) isomer, but not the (+) isomer, of naloxone blocked binding of [125 I][Met 5]-enkephalin. Third, the specificity of the relationship of the ligand to the binding fragments was demonstrated by the blocking of reaction with the addition of cold [Met 5]-enkephalin. Fourth, in keeping with earlier results showing the temporal relationship of receptor expression, binding polypeptides were

recorded in the developing but not the adult cerebellum. Thus, the biological, pharmacological, and biochemical characteristics of the binding fragments appeared to be consistent with the detection of the binding polypeptides associated with the receptor to OGF.

Using binding fragments of the putative receptor to OGF identified with NEPHGE gels of the P1 fraction of 6-day-old rat cerebellum and [125 I][Met 5]-enkephalin, polyclonal and monoclonal antibodies were produced [133]. In the case of the polyclonal antibodies, for example, immunoblots of nuclear fractions (P1) from developing rat cerebellum were found to react to the polyclonal antibodies generated against a number of binding fragments, with bands of 32, 30, 17, and 16 kDa recorded (Fig. 5); these bands were identical in molecular weight to those detected by ligand blotting. No immunoreactivity was recorded with Western blots of adult rat cerebellum. Subcellular fractionation studies and immunoblotting with polyclonal antisera to the receptor binding polypeptides demonstrated that positive immunoreactivity was confined to the nuclear fraction (P1), and little or no immunoreactivity was recorded in the membrane fraction (P2), microsomal fraction (P3), or soluble fraction (S3). Immunoblotting of two-dimensional NEPHGE gels with polyclonal antibodies revealed staining of 32-, 30-, 17-, and 16-kDa polypeptides; each binding fragment had migrated to the same pH as found earlier in ligand blotting studies. The ability of the antibodies to inhibit the binding of [125 I][Met 5]-enkephalin in P1 preparations was ascertained. At the concentrations tested, these antibodies were found to block specific binding of [125 I][Met 5]-enkephalin (e.g. addition of monoclonal antibody 117-3x revealed $41 \pm 4\%$ of the binding of controls). In the case of the monoclonal antibody, addition of γ -globulin did not influence the binding of radiolabeled [Met 5]-enkephalin to the receptor ($93 \pm 4\%$ of control binding). To elucidate whether the blocking of receptor binding was related to non-specific binding by the Fc portion of the IgG, IgG fractions of some antibodies were digested with pepsin to generate F(ab') $_2$ fragments. The F(ab') $_2$ fragments were able to block binding of [125 I][Met 5]-enkephalin to the receptor ($47 \pm 5\%$ of control binding). As a final characterization of the antibodies, both polyclonal and monoclonal antibodies were found to immunoprecipitate OGF receptor binding polypeptides. Antibodies to one binding fragment immunoprecipitated all four binding fragments of the receptor (Fig. 6).

Although a distinct receptor for an opioid peptide was discovered and characterized by function, biochemistry, physiology, anatomy, and pharmacology, the central question remained as to what this receptor was composed of at the molecular and protein level. Since the receptor had the pharmacological attributes of an opioid receptor: naloxone sensitivity and stereospecificity, another issue revolved around the relationship of this unique receptor to classical opioid receptors. Indeed, many of the doubts about the

NEPHGE

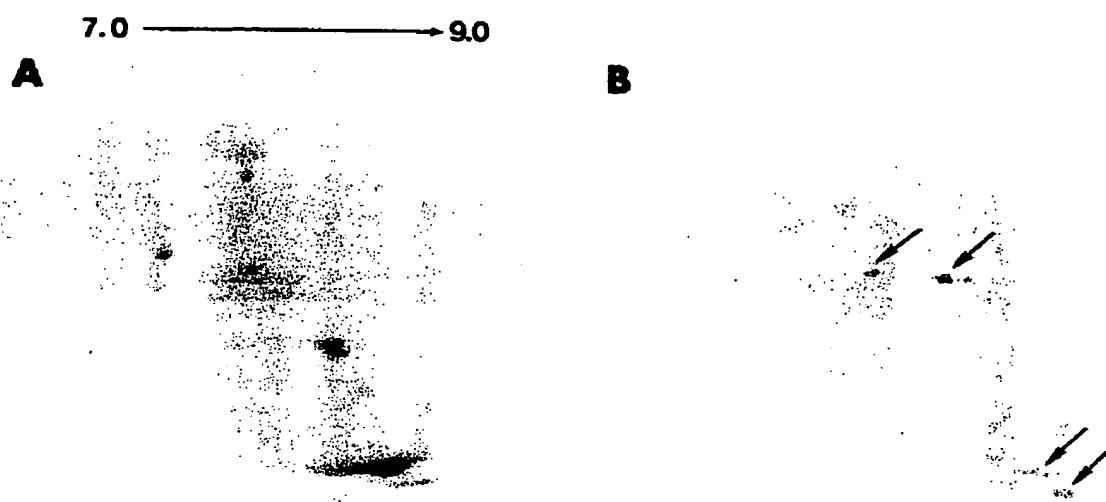


Fig. 4. Preparations of P1 from the 6-day-old rat cerebellum evaluated by two-dimensional non-equilibrium pH gel electrophoresis (NEPHGE)-SDS-PAGE and stained with Coomassie blue (A) or electrotransferred to nitrocellulose and ligand blotted with [125 I][Met 5]-enkephalin (B). The position of the 32-, 30-, 17-, and 16-kDa binding fragments are denoted by arrows (from Zagon et al. [97], with permission).

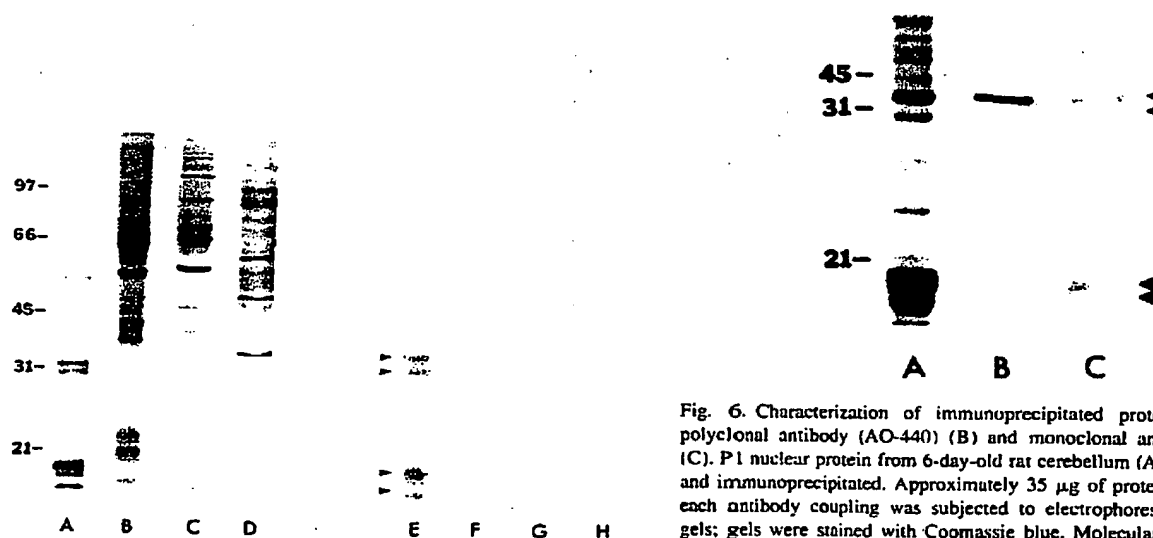


Fig. 5. Western blotting of 6-day-old rat cerebellum fractionated through a sucrose density gradient, prepared for SDS-PAGE, and stained with Coomassie blue (A–D), or electroblotted to nitrocellulose and incubated with polyclonal antibody AO-440 generated against a 17-kDa binding fragment of the receptor for OGF. Lanes A, E, nuclear fraction (P1); B, F, membrane fraction (P2); C, G, microsomal fraction (P3); D, H, soluble fraction (S3) supernatant. Note that immunoreactivity is demonstrated only in the P1 nuclear fraction. Molecular weight markers ($\times 10^3$ Da) are indicated in the left column. The position of the 32-, 36-, 17-, and 16-kDa binding fragments are denoted by arrowheads from Zagon et al. ([133], with permission).

Fig. 6. Characterization of immunoprecipitated proteins by both a polyclonal antibody (AO-440) (B) and monoclonal antibody (117-3X) (C). P1 nuclear protein from 6-day-old rat cerebellum (A) was solubilized and immunoprecipitated. Approximately 35 μ g of protein obtained from each antibody coupling was subjected to electrophoresis on 12% SDS gels; gels were stained with Coomassie blue. Molecular weight markers are shown in the left lane ($\times 10^3$ Da) (from Zagon and McLaughlin [133], with permission).

classification of this receptor could be discerned by the tentative naming of the receptor, the ζ opioid receptor, in deference to the pharmacology but in spite of practically all other characteristics. What needed to be resolved were the molecular instructions for this receptor. The antibodies to the OGF binding fragments and our knowledge about the temporal and spatial distribution will be seen as

merging together to decipher the molecular biology of the receptor to OGF.

6. Molecular biology of the OGF receptor

6.1. Rat OGF_r

Using antibodies to the receptor for OGF, a λ gt11 expression cDNA library constructed from 18-day-old fetal rat brain mRNA was screened to identify clones encoding OGF binding proteins [150]. A partial cDNA was identified by this approach, and used to find a full-length cDNA sequence by hybridization screening. The double-stranded DNA was isolated and sequenced, and found to be a 2.1-kb cDNA that encoded a protein of 580 amino acids, with eight imperfect repeat units of nine amino acids each at positions 467–538 (Figs. 7, 8). The molecular weight as calculated from the sequence was 58 kDa. A search of the sequence in GenBank revealed no homology to this cDNA, and the nucleotide sequence was deposited in GenBank under accession number AF156878. A search through a series of databases for motifs showed that only the motif for a nuclear localization signal (NLS) was recognizable (Fig. 9).

6.1.1. Pharmacology of rat OGF receptor

To confirm the cloned cDNA for the OGF binding protein, fusion proteins were generated and receptor binding analysis with [3 H][Met⁵]-enkephalin was performed (Fig. 10). Specific and saturable binding was observed, with a mean binding affinity (K_d) of 2.8 ± 1.1 nM and binding capacity (B_{max}) of $10\,530 \pm 2237$ fmol/mg protein. Addition of naltrexone to the preparations significantly reduced specific and saturable binding, with reductions in B_{max} of 83% noted. Representative Scatchard plots of specific binding of [3 H][Met⁵]-enkephalin revealed a one-site model of binding. Using a variety of ligands that recognized classical opioid receptors, no competitive binding ($>10^{-3}$ M) for radiolabeled [Met⁵]-enkephalin by DAMGO or morphine sulfate (μ receptor), DPDPE (δ receptor), and dynorphin A1-8 and U69,583 (κ receptor) was observed.

6.1.2. Western blotting and immunocytochemistry of rat OGF receptor

Antibodies to the recombinant fusion protein were generated, and a 1:1000 dilution of antisera detected 10 ng of fusion protein. When reacted with nuclear preparations of 6-day-old rat cerebellum in one-dimensional Western blots, these antibodies recognized a 62-kDa full length OGF_r protein, as well as the four binding fragments: 32-, 30-, 17-, and 16-kDa polypeptides; in addition the antibodies detected the recombinant protein. These antibodies were used in immunocytochemistry and found to stain

intensely the external germinal layer of the 6-day-old rat cerebellum, but little immunoreactivity was detected in the adult rat cerebellum; this staining pattern resembled that observed with antibodies to authentic binding protein.

6.1.3. Antisense studies

To study the function of the isolated cDNA, a 23-mer antisense S-ODN (nuclease-resistant phosphorothioate) targeted against a sequence containing the translation initiation site of the OGF binding protein was designed and added to log-phase cultures of IEC-6 rat intestinal epithelial cells (Fig. 11). This oligoprobe had no homology to sequences for rat in a search of databases. The antisense S-ODN elevated cell number by 294% from control cultures within 48 h of exposure. The cell cultures treated with the scrambled probe as a control were similar in growth to control levels. Cultures treated with naltrexone, an opioid antagonist to the opioid binding protein, also demonstrated increased cell growth from control values; the naltrexone group had 223% more cells than control cultures.

These studies presented a number of pieces of evidence that form a persuasive argument that the molecular nature of the receptor for OGF has been identified. First, pharmacological studies showed that the translated protein for the OGF_r exhibited high-affinity binding that was specific, saturable, and consistent with a one-site model of binding. This binding could be displaced by the opioid antagonist, naltrexone, suggesting that the binding reaction was at the level of an opioid-like receptor. Binding to an endogenous opioid peptide, OGF, was recorded, but synthetic and natural ligands for classic opioid receptors such as μ , δ , and κ were not competitive, indicating that OGF had special properties in recognizing the translated protein. These pharmacological characteristics of the recombinant protein are consistent with those reported in earlier studies with cells and tissues (see above for a review). Second, the temporal appearance of the mRNA and the translated protein of the OGF_r revealed greater expression in developing neural tissues than adult counterparts. Moreover, the temporal patterns of gene and protein expression were directly related. The prevalence of the receptor in developing rather than in postmitotic cells in adult tissue is consonant with information in earlier reports showing that OGF activity was targeted to cells that were developing, carcinogenic, renewing, or repairing. Third, the spatial distribution of the OGF_r showed that it was associated with developing neural cells but not their adult counterparts. Subcellular fractionation studies and immunocytochemical findings in cells and tissues have recorded a similar structural relationship, and the observation that staining of the OGF_r was associated with the cytoplasm of the cells indicates that the antibody recognized sites of synthesis as well as assembly. Fourth, antibodies to the OGF_r fusion protein recognized the four binding poly-

Fig. 7. Comparison of amino acids in human, mouse, and rat OGF_r. Identities are indicated by asterisks, conservative substitutions are indicated by colons, and semi-conservative substitutions are indicated by periods. Mismatches are depicted as blank spaces.

Mouse Repeats

- | | | |
|-----|-----------|---|
| 1. | GPEDPKSQV | * |
| 2. | GPEDPKSQV | * |
| 3. | GPEDPKSQV | * |
| 4. | GPEDPKSQV | * |
| 5. | GPEDPKGQV | |
| 6. | EPEDPKGQV | |
| 7. | GPEDPKGQV | |
| 8. | GPEDPKGQV | |
| 9. | GPEDPKSQV | * |
| 10. | GPEDPKSQV | * |
| 11. | EPEDPKSQV | |
| 12. | EPEDPKSQV | |
| 13. | EPEDPKSQV | |
| 14. | GPEDPQSQV | |

gPEDPKsQV Consensus

Rat Repeats

- | | | |
|----|-----------|---|
| 1. | GPEDSNSQV | |
| 2. | GAEDSKSQV | |
| 3. | GPEDPNSQV | * |
| 4. | GLEDPNSQV | |
| 5. | GPEDPNSQV | * |
| 6. | GPEDPNSQV | * |
| 7. | GPEDPNSQV | * |
| 8. | GPEDPNSQV | * |

GpEDpnSQV Consensus

Human Repeats

- | | | |
|----|------------------------|--|
| 1. | SPSETPGPSPAGPAGDEPAE | |
| 2. | SPSETPGPRPAGPAGDEPAE * | |
| 3. | SPSETPGLRPAGPAGDEPAE | |
| 4. | TPSETPGPSPAGPTRDEPAE | |
| 5. | SPSETPGPRPAGPAGDEPAE * | |
| 6. | SPSETPGPRPAGPAGDEPAE * | |
| 7. | SPSETPGPSPAGPTRDEPAK | |

sPSETPGprPAGPagDEPAe Consensus

*** Matches Consensus**

Fig. 8. Amino acid repeats in mouse, rat, and human OGF_r. Asterisks denote identity to the consensus sequence.

peptides/proteolytic fragments (i.e. 32, 30, 17, and 16 kDa) observed earlier, as well as a 62-kDa protein consistent with the full length protein (i.e. translation of the open reading frame). A 62-kDa protein also was sometimes seen in preparations of native homogenates, but was

frequently obscured by β -mercaptoethanol contamination. Whether some or all of the binding polypeptides are proteolytic breakdown products or the result of processing of the 62-kDa protein is presently unclear. Fifth, cell cultures treated with antisense oligonucleotides to the OGF_r exhibited marked increases in the number of cells in contrast to control cultures. The magnitude of change in cell number invoked by the addition of the antisense oligonucleotides was of the order occurring when endogenous opioid peptides were blocked from interacting with their receptors using naltrexone, suggesting that only the receptor now identified for OGF was involved with growth. Thus, although naltrexone blocks classical opioid receptors as well, this opioid antagonist can be used to selectively disrupt the OGF–OGF_r pathway for growth.

The OGF_r tentatively identified earlier in pharmacological and physiological studies as the ζ receptor was different from other classical opioid receptors and originally named to distinguish its special characteristics (i.e. function, spatial and temporal expression, ligand specificity, subcellular location). Moreover, the ζ receptor did share some of the pharmacological and physiological hallmarks of opioid receptors such as reversibility by naloxone and stereospecificity. Comparison of the molecular information for the OGF receptor to that of the opioid receptor family (μ , δ , κ , and ORL/nociceptin/orphanin FQ) shows no structural homology. Indeed, the OGF receptor lacked such characteristics of classical opioid receptors such as a coupling through pertussis toxin-sensitive G-proteins, the similar general structure of an extracellular N-terminal region, seven transmembrane domains and an intracellular C-terminal tail structure. Thus, in view of such pharmacological, physiological, biochemical, anatomical, and molecular differences, the receptor tentatively identified as the ζ receptor was renamed the OGF receptor (OGF_r) (see below for further discussion).

6.2. Human OGF_r

The presence and interaction of OGF–OGF_r have been found to occur in a wide variety of human cells and tissues, including embryos, head and neck squamous cell carcinoma, pancreatic adenocarcinoma, colon cancer, renal cancer, neuroblastoma, skin, corneal epithelium—homeostasis and wound healing, and the gastrointestinal tract. To explore the molecular nature of the OGF receptor in humans, primers generated for the rat cDNA for OGF_r were used with reverse transcriptase-polymerase chain reaction (RT-PCR) and RNA from human placenta—a rich source of receptors for OGF as determined by receptor binding assays [151]. The internal PCR product from the human OGF_r was used as a platform to identify a complete open reading frame through a combination of 5'- and 3'-RACE. Several alternative 3' ends were identified by 3' RACE. The structure of the clone originally identified as 'clone 4' (deposited under accession number AF172451),

Bipartite Nuclear Localization Signal

consensus	*****.*****.*** *****.*** *****.*****.* ..
hu-OGFr	251 QSALDYFMFAVRCRHQRRQLVHFAWEHFRPRCKFVWGPDKLRRFKPSL
mu-OGFr	241 QSALDYFLFAVRCRHQRREL VHF AWEHFKPRREFVWGPRDKLRFRPQTI
rt-OGFr	241 QSALDYFLFAVRCRHQRREL VYFAWEHFKPRREFVWGPRDKLRRFKQTI

bNLS

BB(N₁₀)(3/5B)

Fig. 9. Multiple alignment of human, mouse, and rat OGFr bipartite nuclear localization signals (bNLS; BB(N₁₀)(3/5B)). Each of the OGFr sequences contains a bNLS in an analogous position (amino acids in bold). The rat and mouse sequences contain a second bNLS overlapping the first (underlined) (from Zagon et al. [152], with permission).

encoding an open reading frame of 677 amino acids, has been confirmed by the Human Gene Project and the human EST database (Fig. 8). This clone contained seven imperfect repeats of 20 amino acids each (Fig. 12). The predicted initiation site was flanked by a strong Kozak consensus sequence. The alternatively spliced forms identified by 3' RACE are depicted in Fig. 13. Two alternatively spliced forms are missing the imperfect repeats (deposited in GenBank under accession numbers AF172449 and AF172450). Expressed sequence tags (EST) have been noted for the clone containing 677 amino acids, but not for the other two forms of the OGF receptor, perhaps indicating that these shorter forms are PCR artifacts. Two previously reported alternative forms are clearly the result of a PCR artifact. The cDNA designated 'clone 8' has one more copy of the repeat than is present in the human genome sequence (deposited in GenBank under

accession number AF172453). A second clone ('clone 7') is missing one copy of the repeat relative to the genome, and there are no signals suggesting this is an alternatively spliced form (deposited in GenBank under accession number AF172452). Extensive searches of databases have revealed no recognizable protein domains for human OGFr, with the exception of a motif for a NLS (Fig. 9).

Northern blot analysis of human fetal and adult tissues, as well as cancer tissues and cell lines, was performed with radiolabeled cDNA for OGFr to begin to examine diversity of this receptor in humans (Fig. 14). In human fetal tissues, transcript sizes of 1.7 and 2.4 kb were observed, whereas in adult tissues, cancer cell lines and oncogenic tissues only a 2.4-kb mRNA was detected.

Finally, in order to study the function of the isolated cDNA, a 23-mer antisense S-ODN was designed and added to log-phase cultures of SK-N-AS human neuroblastoma cells (Fig. 15). The antisense S-ODN elevated cell number by 60% from control cultures within 48 h of exposure. The cell cultures treated with the control scrambled oligonucleotides were similar in growth to control levels. These experiments were extended in another human neuroblastoma cell line—SK-N-SH. Our investigation (Zagon and McLaughlin, unpublished observations) showed that cells transfected with 23-mer antisense S-ODN and grown for 72 h had 34% more cells than controls (wild-type and scrambled probe); this difference between the antisense and control cultures was statistically significant ($P < 0.001$). Examination of immunocytochemical-stained preparations of these 72-h cell cultures (antisense, wild-type, scrambled) with antibodies to OGFr revealed that the antisense-treated cultures had threefold less intensity of staining than control or scrambled cells using densitometric measurements; this difference was significant at $P < 0.001$.

To confirm the cloned cDNA for the human OGFr, a full-length fusion protein was used in receptor binding analysis with [³H][Met³]-enkephalin (Fig. 16). Specific and saturable binding of the purified recombinant protein

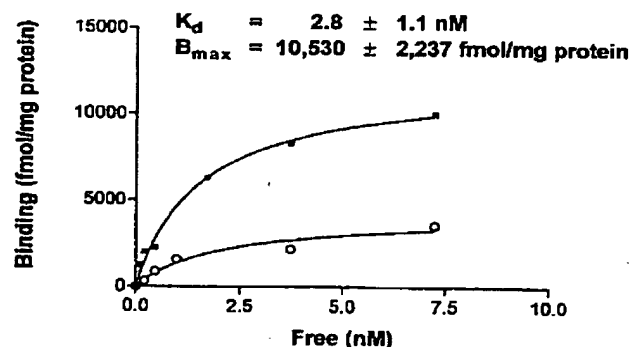


Fig. 10. Representative saturation isotherm of specific binding of [³H][Met³]-enkephalin (■) to purified fusion protein translated in vitro from the rat OGFr cDNA clone #12. Mean \pm S.E.M. binding affinity (K_d) for six assays was 2.8 ± 1.1 nM and binding capacity (B_{max}) was $10,530 \pm 2,237$ fmol/mg protein. Binding was significantly reduced with the addition of 1μ M concentrations of the opioid antagonist naltrexone (○). Representative Scatchard plot of specific binding of radiolabeled [Met³]-enkephalin revealed a one-site model of binding (data not shown) (from Zagon et al. [150], with permission).

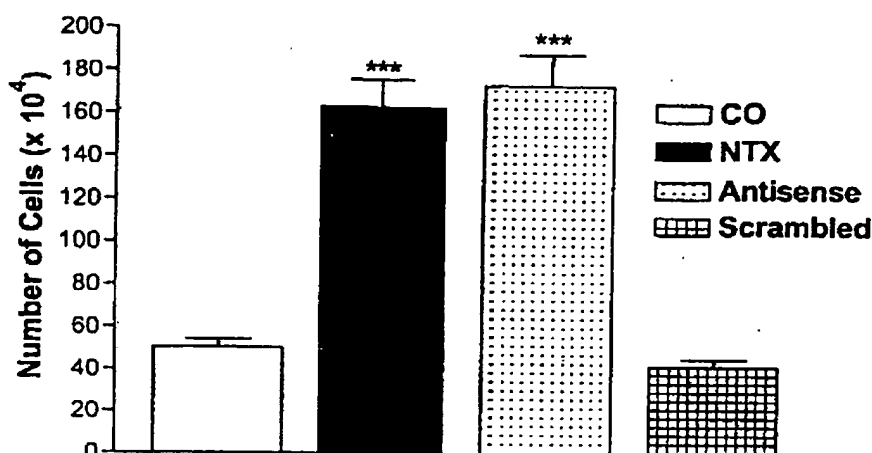


Fig. 11. Histogram of cell number in cultures of IEC-6 rat intestinal epithelial cells treated for 48 h with either sterile water (CO), 10^{-6} M NTX, 10^{-6} M 23-mer S-ODN (Antisense), or scrambled oligoprobe (Scrambled). Cells (5×10^5) were plated and compounds and fresh media added 24 and 48 h later. After 72 h in culture, cells were trypsinized, stained with trypan blue, and counted with a hemacytometer. Data represent mean \pm S.E.M. ***Significantly different from CO at $P < 0.001$ (from Zagon et al. [150], with permission).

was observed, with a mean K_d of 6.6 ± 3.2 nM and a B_{max} of $21\,300 \pm 5000$ fmol/mg protein. Recombinant glutathione-S-transferase (GST) alone did not bind radiolabeled [Met⁵]-enkephalin in a specific and saturable manner, and was considered background.

6.3. Mouse OGF_r

In order to formulate a broader perspective of the structure of OGF_r, a study was conducted to explore the molecular organization of OGF_r in mouse [152]. The relationship of OGF–OGF_r in the mouse is of special interest as the mouse has been used to examine homeostatic cellular renewal and wound healing, and will serve as the model for transgenic mutants. Using the rat OGF_r cDNA in a BLAST query, an EST sequence from a 14-day-old mouse embryo was identified. This cDNA, which contained a complete open reading frame was sequenced in its entirety. The nucleotide sequence, and the deduced amino acid sequence, of the full length cDNA, along with portions of the 5' and 3' untranslated regions are presented in Fig. 7. The open reading frame was found to encode a protein of 634 amino acids, with 14 imperfect repeat units of nine amino acids each at positions 467–592 (Fig. 8). The molecular weight as predicted from the sequence was 71 kDa, and the protein had a predicted isoelectric point of 4.5. Examination of the content of amino acids showed that glutamic acid and aspartic acid accounted for 11.4 and 8.5%, respectively, of OGF_r. Additionally, an abundance of proline (61 prolines in total that accounted for 9.4% of OGF_r) were generally positioned in the C-terminal end of the protein. The nucleotide sequence of mouse OGF_r was deposited in

GenBank under accession number AF303894.

Using a variety of sequence analysis tools including ProDom, PROCITE, MOTIFS, PFAM, and COILS, no significant homologies to known domains were recognized. The only functional motif found for mouse OGF_r was a bipartite NLS (Fig. 9).

Northern blot analysis of adult mouse tissues was performed with radiolabeled cDNA for the mouse OGF_r in order to inspect tissue distribution. Mouse OGF_r was observed in all six organs examined in the adult animal: brain, heart, lung, liver, kidney, and skeletal muscle, with a transcript size of 2.1 kb recorded in all tissues.

6.4. Comparison of OGF_r structure in mouse, rat, and human

Alignment of the amino acids for mouse, rat, and human OGF_r is presented in Fig. 7. As noted in a schematic summary in Fig. 17, the greatest identity and similarity for the sequence of amino acids in OGF_r for mouse, rat, and human occurred at the N-terminus. As one progressed towards the C-terminus, particularly in the last one-third of the receptor, the amino acid sequence between species showed an identity ranging from 23% between human and rat and 31% between human and mouse, and similarities in identity and similarity of approximately 45% between human and mouse or rat. The relatively low identity between rodent and human raises the question of whether OGF_r is present as paralogues (i.e. family members) or orthologues (i.e. the same gene in different species). Given that there are no other sequences in the databases related to OGF_r in any of the species examined, the best argument to date would be for the case of orthologues.

Fig. 12. Nucleotide and predicted amino acid sequences of human OGFr cDNA (clone #4); 5'- and 3'-untranslated regions are included. Repeats are denoted by single and double underlining.

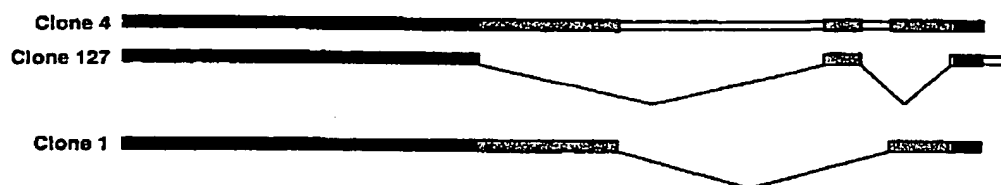


Fig. 13. Alternatively spliced forms of human OGFr cDNA. Black indicates identical in all three splice variants, gray is identical in two of the three forms, and white is unique.

A significant feature of the amino acid sequence of mouse, rat, and human OGFr was the finding of repeated sequences (Fig. 8) localized in the C-terminus. The amino acid content—and sequence—of the consensus of these repeats in mouse and rat were striking in resemblance, with glycine–proline–glutamic acid–aspartic acid–proline–lysine (mouse)/asparagine (rat)–serine–glutamine–valine, being the most common. The seven repeats in the human sequence differed considerably from mouse/rat. One sur-

prising feature of these repeats is that, despite the differing lengths of the repeats (20 amino acids in humans and nine amino acids in mouse/rat), 22 prolines have identical positioning in rat, mouse, and human. In fact, 16 other prolines in all species were in similar positions. Because

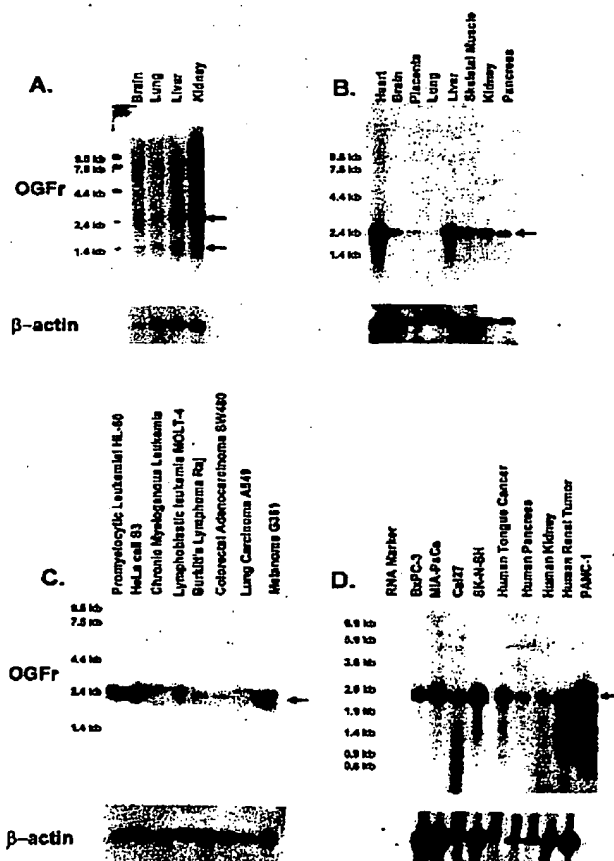


Fig. 14. Northern blot analysis of the receptor for OGF in human fetal (A) and adult (B, D) tissues, cancer cells and tissues (C, D), and normal pancreas and kidney; corresponding β -actin levels are shown below each blot (from Zagon et al. [151], with permission).

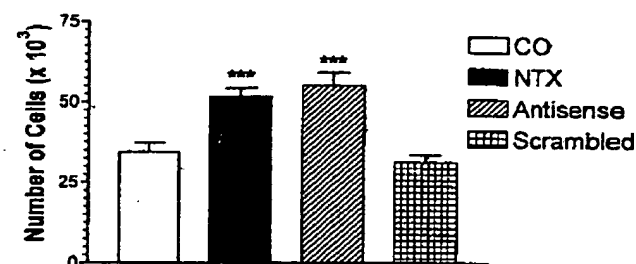


Fig. 15. Histogram of cell number in cultures of SK-N-AS human neuroblastoma cells treated for 48 h with either sterile water (CO), 10^{-6} M NTX, or 10^{-6} M antisense or scrambled oligonucleotide. Cells (6×10^5) were plated and compounds and fresh media added 24 and 48 h later. After 72 h in culture, cells were trypsinized, stained with trypan blue, and counted with a hemacytometer. Data represent mean \pm S.E.M. ***Significantly different from CO at $P < 0.001$ (from Zagon et al. [151], with permission).

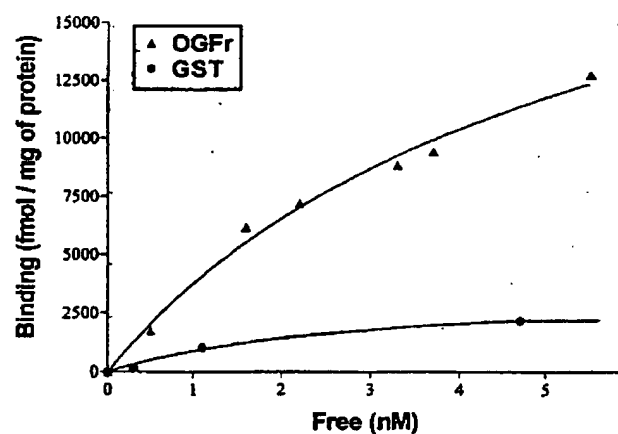


Fig. 16. Representative saturation isotherm of specific binding of [3 H][Met⁵]-enkephalin to purified full-length fusion protein translated in vivo from the human OGFr cDNA (Δ), or the recombinant GST alone (\bullet). Mean \pm S.E.M. for three assays revealed a K_d of 6.6 ± 3.2 nM and the B_{max} was $21\,300 \pm 5000$ fmol/mg protein.

Mouse, Human, and Rat OGF _r							
Mouse	1	287	434	633			
	<table><tr><td>78% Identical 84% Similar</td><td>59% Identical 63% Similar</td><td>31% Identical 34% Similar</td></tr></table>				78% Identical 84% Similar	59% Identical 63% Similar	31% Identical 34% Similar
78% Identical 84% Similar	59% Identical 63% Similar	31% Identical 34% Similar					
Human	1	297	464	677			
	<table><tr><td>79% Identical 84% Similar</td><td>56% Identical 60% Similar</td><td>33% Identical 35% Similar</td></tr></table>				79% Identical 84% Similar	56% Identical 60% Similar	33% Identical 35% Similar
79% Identical 84% Similar	56% Identical 60% Similar	33% Identical 35% Similar					
Rat	1	287	434	580			
Amino Acid Number							

Fig. 17. Comparison of amino acid similarity between mouse, human, and rat OGF_r. The amino acid similarities between mouse and human, and rat and human, were inconsistent throughout the OGF_r, and were higher at the N-terminus. Numbers above or below boxes indicate amino acid position of the boundaries determined by inspection.

prolines are known to constrain the folding of proteins, it may be that the specific placement of prolines in the OGF receptor dictate a structural conservatism that is necessary for this receptor to function. Additionally, the OGF_r protein appears to have an abundant acid content with over 20% of the receptor in mouse, for example, containing either glutamic acid or aspartic acid. Whether the acid nature of OGF_r belies a transcription factor is not yet known.

As mentioned above, the only recognizable sequence motif is a bipartite NLS in OGF_r of all three species (Fig. 9), with both mouse and rat OGF_r having second, overlapping bipartite nuclear localization signals. This sequence was defined according to published criteria that included: (i) two adjacent basic amino acids (Arg or Lys), (ii) a spacer region of any 10 residues, and (iii) at least three

basic residues (Arg or Lys) in the five positions after the spacer region [10,69]. The significance of the NLS to nucleocytoplasmic shuttling is discussed later.

7. The OGF receptor gene and chromosomal location

The gene for the human OGF receptor has been filed in GenBank (AF112980) (Fig. 18). The gene is at least 9 kb in length, and consists of seven exons and six introns. The splicing pattern has been predicted, and each of the introns had the canonical GT-intron-AG structure. The sizes are listed in nucleotides as follows:

Exon 1	178 +	
Intron A		2503
Exon 2	74	
Intron B		642
Exon 3	81	
Intron C		1260
Exon 4	81	
Intron D		836
Exon 5	71	
Intron E		897
Exon 6	152	
Intron F		620
Exon 7	1702 +	

Using fluorescence in situ hybridization, the chromosomal location of the human OGF receptor was determined to be 20q13.3 (Fig. 19). A search of the Genome Database showed that this chromosomal location also contained genes for endothelin 3, laminin (alpha 5), collagen (type IX, alpha 3), and neuronal nicotinic acetylcholine receptor. Chromosomal translocations and aberrations at 20q13.3 have been related to autism, an inherited epilepsy of newborns, as well as myeloproliferative disorders.

Splicing Pattern of OGF_r

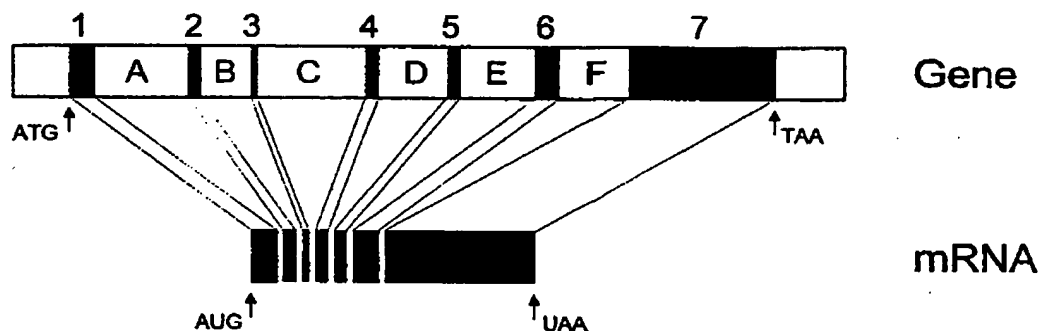


Fig. 18. The gene and splicing pattern for the longest cDNA related to the human OGF_r. See text for sizes of introns and exons.

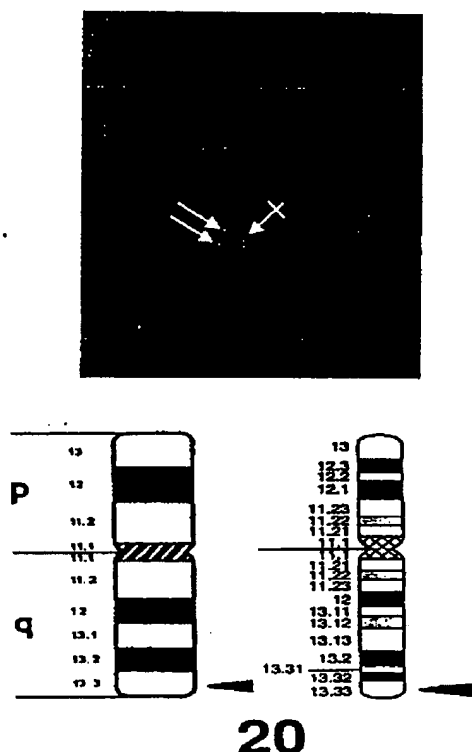


Fig. 19. FISH preparation and a companion ideogram (from the International System for Human Cytogenetic Nomenclature, 1995) showing the localization of OGFr to chromosome 20q13.3 (arrow) (from Zagon et al. [151], with permission).

8. The OGF receptor and classical opioid receptors

Given that the opioid peptide—OGF—has been demonstrated to be the peptide associated with OGFr and that the system modulates growth, the relationship of OGFr to classical opioid receptors should be reviewed. Two criteria have been used in the past for classification of an 'opioid' effect mediated by an opioid receptor: naloxone reversibility and stereoselectivity [39,63]. The concept that the opioid receptor recognizes opioids (natural and synthetic) and demonstrates naloxone reversibility and stereoselectivity (i.e. the (–) isomer is more active than the corresponding (+) derivative) was expanded by the evidence of the existence of multiple opioid receptors (e.g. Ref. [44]). It now is clear that there are at least three well-defined or 'classical' types of opioid receptors: μ , δ , and κ receptors, and in the past decade genes encoding these receptors have been cloned [6,12,31,55,56,88]. The nomenclature for opioid receptors remains unclear, with the μ receptor termed MOR, OP₃, and MOP, the δ receptor termed DOR, OP₁, and DOP, and the κ receptor termed KOR, OP₂, and KOP [2,61].

Analysis of the sequences of these cloned opioid receptors revealed that they belong to the super-family of G

protein-coupled receptors (GPCR) and the sub-family of rhodopsin receptors [36,37,68]. All three cloned receptors have the putative structure of seven transmembrane domains, an extracellular N-terminus with multiple glycosylation sites, a third intracellular loop with multiple amphiphatic α -helices, and a fourth intracellular loop formed by the putative palmitoylation sites at the carboxyl terminus [6,12,13,31,41,54,88]. In general, there is a 60% identity between these receptors, with the μ receptor being 66% identical to the δ receptor and 68% identical to the κ receptor, whereas the δ and κ receptors have 58% identical amino acid sequences [36,72]. The greatest homology is found in the transmembrane domains (73–76%) and intracellular loops (86–100%). These opioid receptors are capable of regulating the same second messengers, with activation of the receptors causing inhibition of adenylyl cyclase activity [6,12,13,31,41,54,88] and L-type [64] and N-type [76] Ca²⁺ channels.

More recently, the identification of a novel receptor that bears a 65% identity to the molecular structure of classical opioid receptors has come forth [19,56]. The receptor was detected in rat, mouse, and man, with a 90% or greater degree of homology among the species variants. This receptor, termed ORL1, NOR, and NOP, does not bind opioid ligands with high-affinity, but recognizes the endogenous 17-amino acid peptide termed orphanin FQ/nociceptin (OFQ/N₁₋₁₇) in a naloxone-insensitive manner.

Thus, it is interesting that the endogenous opioid peptide, [Met⁵]-enkephalin, can function as a neurotransmitter/neuromodulator by way of classical opioid receptors, and it can also function in growth—and does so by way of a totally different receptor—OGFr. The interaction of OGF with OGFr obeys the pharmacological principles of the definition of an opioid receptor, yet unexpectedly the OGF receptor has no homology to the opioid receptors in terms of nucleotides or amino acids. This stands in contrast to the orphanin FQ/nociceptin receptor, ORL1/NOR/NOP which resembles the molecular structure of classical opioid receptors, yet its pharmacology shows little relationship to opioid receptors.

9. Location and distribution of the OGF receptor and OGF

Using polyclonal antibodies to the OGF receptor, frozen sections of the 6-day-old rat cerebellum were examined in order to localize receptor distribution (Fig. 20). A progressive diminution in fluorescence from the pial surface inward was recorded. The external germinal layer was brightly fluorescent, the molecular layer somewhat reactive, and the internal granule and medullary layers only slightly reactive. Higher magnification showed the perinuclear region of the external germinal cells to be stained intensely, but little immunoreactivity could be discerned in

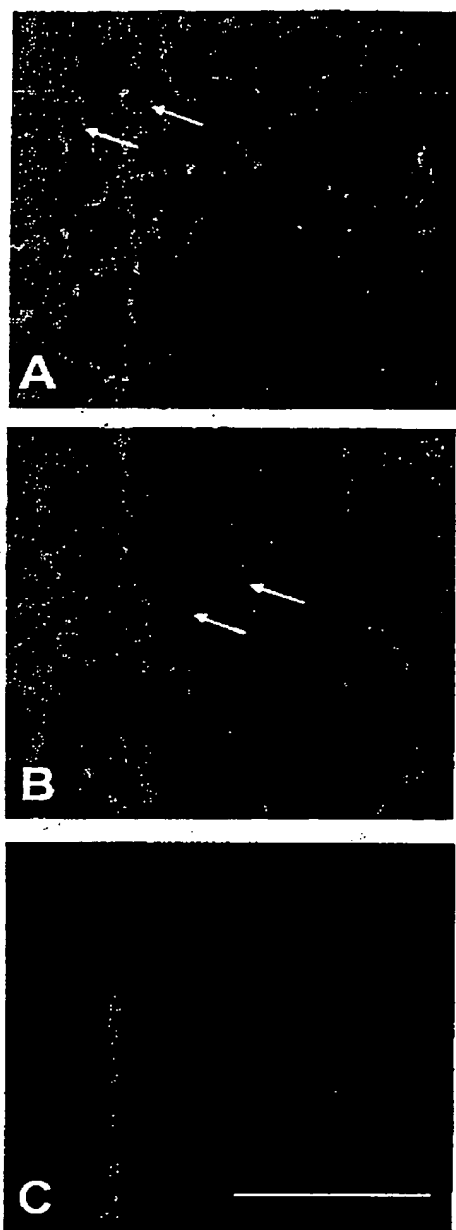


Fig. 20. Distribution of OGF in external germinal cell layer in adjacent sections of 6-day-old rat cerebellum (A, B) as detected by an antibody to an OGF fusion protein (A) or an antibody to the native 17-kDa binding fragment (B); the pial surface is located in the upper right of the section. Internal granule cells in adult rat cerebellar sections processed with antibody to the fusion protein antibody (C) or the native binding fragment (data not shown) revealed no immunoreactivity. Arrows = OGF immunoreactivity of external germinal cells. Bar = 50 μ m (from Zagon et al. [150], with permission).

the nucleus. Cells near the pial surface usually seemed to stain more intensely than those adjacent to the molecular layer. The nature of the immunoreactivity in the molecular

layer with respect to an association with cellular elements was difficult to decipher, although Purkinje cells appeared to be fluorescent. The internal granule layer exhibited a mosaic pattern of staining, with immunoreactivity related to a small proportion of cells. Neurons in the deep cerebellar nuclei, as well as glia in the medullary layer, reacted with the antibody. No specific staining was detected in pre-absorbed controls, pre-immune controls, or in the cerebellar cortex from adult rats. Subsequent studies with antibodies to the OGF receptor showed staining of developing and renewing, but not postmitotic cells, as well as neoplastic tissues and cells [4,40,47,49,53,87,98–100,140–142,144,145,150,154,156]. In larger cells, staining was more intense proximal to the nucleus. The apparent staining in the cytoplasm, but binding activity in the nuclear fraction, can be reconciled in a number of ways. First, the cytoplasm is the site of synthesis of the OGF receptor, and the antibody may be recognizing epitopes on newly synthesized and transported receptor protein. Second, the immunofluorescence peripheral to the nucleus may radiate into the cytoplasm, providing the appearance of cytoplasmic staining. Further studies have shown that the receptor for OGF has been detected in a variety of animal and human cells and tissues.

Recent immunoelectron microscopic studies are now resolving the relationship of the distribution of OGF and OGF by colocalization experiments using a double-face immunogold staining procedure (Fig. 21) (Zagon, Ruth, McLaughlin, unpublished observations). Immunogold for both OGF and OGF can be detected in association with the outer nuclear envelope, and an abundance of OGF–OGF immunoreactivity was found in the perinuclear position. Some gold particles for OGF and OGF were localized in what appeared to be a nuclear pore. Moreover, anti-OGF and anti-OGF IgG could be found in the inner nuclear lamina, and at the interface of heterochromatin and euchromatin. Studies with colocalization of OGF and karyopherin β , which interacts with NLS and plays a role in nucleocytoplasmic shuttling, have demonstrated that these elements are distributed in the perinuclear location, and in the nuclear pore (Zagon, Ruth, McLaughlin, unpublished observations).

10. Diversity of OGF receptor

The OGF receptor has been widely reported in rat, mouse, and human cells and tissues [4,24,40,47,49–51, 53,93,96,98–100,133,140–142,144,145,149,150,154,156]. A study of the diversity of OGF (and OGF) amongst species was undertaken in corneal epithelium [140]. Both peptide and receptor were present in a wide variety of classes of the phylum Chordata, including mammalia, aves, reptilia, amphibia, and osteichthyes. These results suggest that the OGF receptor—and OGF—may have originated as early as 300 million years ago, and the

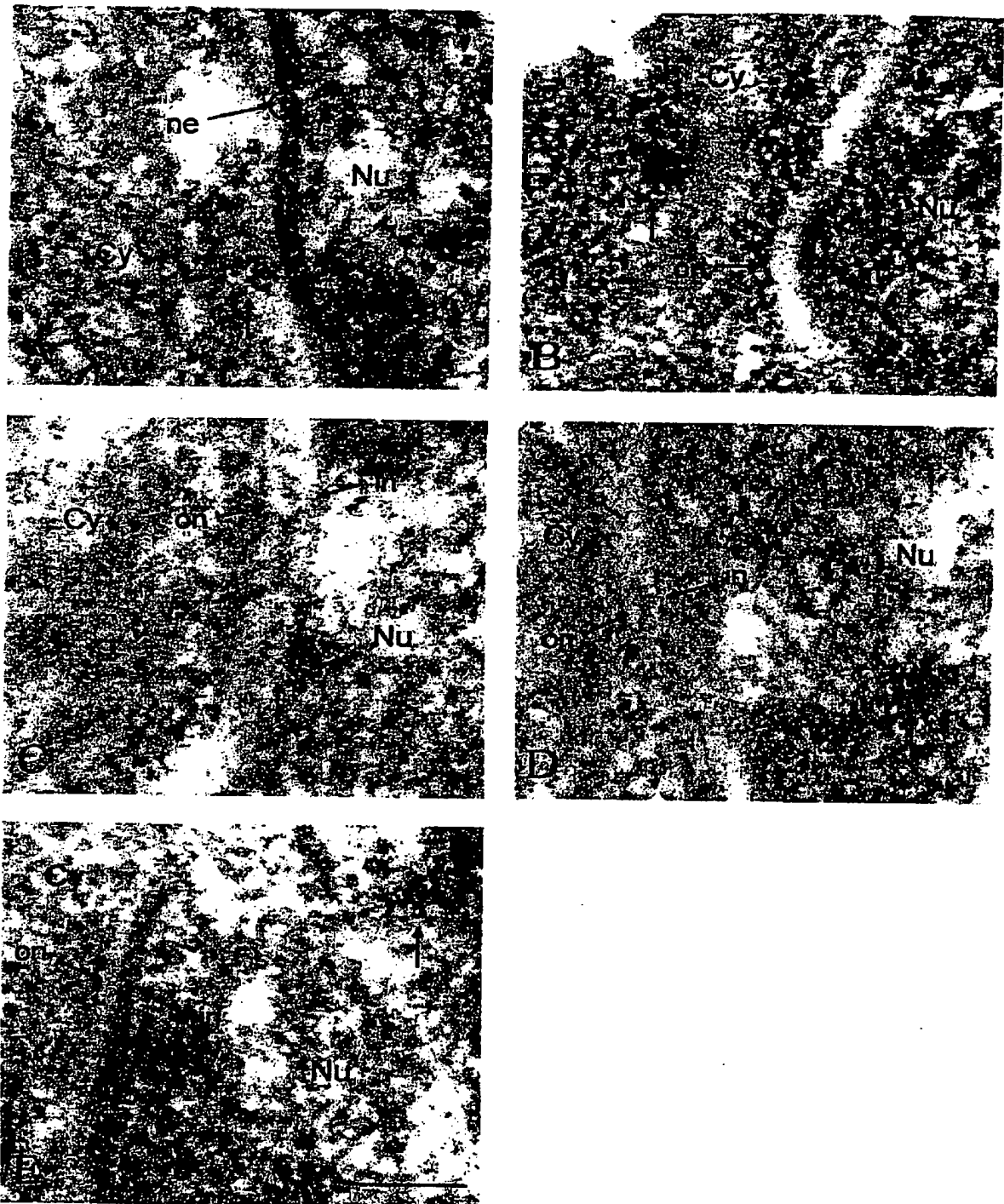


Fig. 21. Immunoelectron micrographs of the rat tongue epithelium stained with antibodies to OGF_r (6 nm gold) and OGF (10 nm gold); a double-face immunogold labeling procedure was used. Colocalization of peptide and receptor (arrows) can be observed on the outer nuclear envelope (A), in the perinuclear cytoplasm (B), traversing the nuclear pore (C), in association with the inner nuclear lamina (D), and at the periphery of heterochromatin aggregations (E). Bar=200 nm.

function of this endogenous opioid system in at least the cellular renewal and homeostasis of the vertebrate corneal epithelium is a requirement.

Although little is known about the origin of the OGF receptor prior to vertebrates, it is fascinating that specific and saturable binding of radiolabeled OGF to the bacterial strain *Staphylococcus aureus* has been recorded [130]. A binding affinity of 1.7 nM and a binding capacity of 202 fmol/mg protein, in a one-site model, has been reported for *S. aureus*. Classical opioid receptors, including μ , δ , and κ , were not detected in these bacteria. Functional studies with OGF showed that this peptide was inhibitory to the growth of *S. aureus*, as well as *Staphylococcus marcesans* and *Pseudomonas aeruginosa*, with this action found to be receptor-mediated. Thus, it would appear that OGF and OGFr may have originated as early as 2 billion years ago, the time corresponding to the evolution of bacteria.

11. Signal transduction and the function of the OGF-OGFr system

Given the enormous amount of information about the OGF receptor already reported, and in light of knowledge about cell and molecular biology concerning nucleocytoplasmic interactions [10,28,46,62,69], the transduction of signaling from OGF-OGFr interaction to modulating cell function merits discussion. At least three clues about the interfacing of OGF with OGFr should be restated: (i) OGF-OGFr modulates DNA synthesis, (ii) OGF and OGFr colocalize on the outer nuclear envelope, in the nuclear pore, and on the inner nuclear lamina and periphery of the heterochromatin, and (iii) the OGF receptor has a nuclear localization signal. Based on such observations above, at least one working model can be put forth (Fig. 22). OGF, presumably transported actively or passively from the extracellular milieu, interacts with OGFr residing on the outer nuclear envelope. Both the peptide and receptor form a complex that appears to be released from the outer nuclear envelope, and accumulates in a perinuclear location. This peptide-receptor complex interfaces directly with importins/karyopherins (Kap) by way of the bipartite NLS of the OGF receptor and is actively imported through the nuclear pore complex (NPC). Presumably this nucleocytoplasmic shuttling takes place in the presence of Ran, and Ran interacting proteins (p10/NTF2). The NPC is approximately 100 nm in diameter and is formed by the juncture of the inner and outer nuclear membranes, with the core of the NPC consisting of a cylindrical assembly of eight identical spoke structures symmetrically arranged around the central transporter; peripherally associated nuclear and cytoplasmic filaments project from the core. Thus, molecules of up to about 9 nm in diameter, corresponding to a globular protein of approximately 60 kDa, can in principle enter or

leave the nucleus by diffusion through the NPC. In theory, because the OGF-OGFr complex is greater than 60 kDa, an active, signal-mediated process by way of the NLS is necessary for nucleocytoplasmic transport. Import is initiated by docking to the cytoplasmic filaments, followed by multiple rounds of interaction between the Kaps-substrate complex and nucleoporins (nups, proteins composing the NPC) closer to the nuclear interior. These multiple docking and undocking events are governed by the actions of Ran and its regulators. Ran, together with several other key Ran-interacting and regulating proteins including nuclear transport factor 2 (NTF2), Ran binding protein 1 (RanBP1), Ran GTPase activating protein 1 (RanGAP1), and the nucleotide exchange factor RCC1, mediates the second energy-dependent transport step of translocation of the OGF-OGFr complex through the NPC, and release into the nucleus. Inside the nucleus, Kap α and β dissociate (are recycled by export to the cytoplasm) from the OGF-OGFr complex in the presence of Ran-GTP. The OGF-OGFr complex appears to associate with the inner nuclear lamina and the periphery of the heterochromatin, whereby DNA replicative activity can be suppressed by this peptide-receptor complex. Testing the various aspects of this model clearly represents the next step in understanding this unique growth regulatory system.

12. Implications for health and disease

As briefly discussed at the beginning of this review, the literature reveals that OGF-OGFr interaction plays a role in cellular renewal in homeostasis, wound healing, development, angiogenesis, and cancer. Assuming that the peptide and receptor are in a delicate equilibrium, as shown by disruption by such experiments as exposure to excess OGF, deprivation of OGF-OGFr interfacing using opioid antagonists such as NTX, neutralization of OGF action using antibodies to this peptide, and antisense studies with oligonucleotides to OGFr, one would predict that alteration in one or both peptide and receptor at the transcription and/or translation levels could have a profound effect on growth. In the case of the OGFr, for example, a number of findings offer provocation as to the fundamental nature of this receptor to biological processes and the drastic repercussions when defects occur. In the clinical setting, tumor specimens of human squamous cell carcinoma of the head and neck (SCCHN), and human pancreatic adenocarcinoma, reveal a subnormal number of receptors; the binding affinity of the OGF receptor was comparable to normal specimens [50,149]. Preliminary examination of gene expression indicates that OGFr mRNA in tumor tissue from patients with SCCHN or pancreatic cancer and samples of normal tissues and samples marginal to the neoplasia are comparable. These results would suggest that defects in either translation and/or post-translation as to the OGF receptor, but not

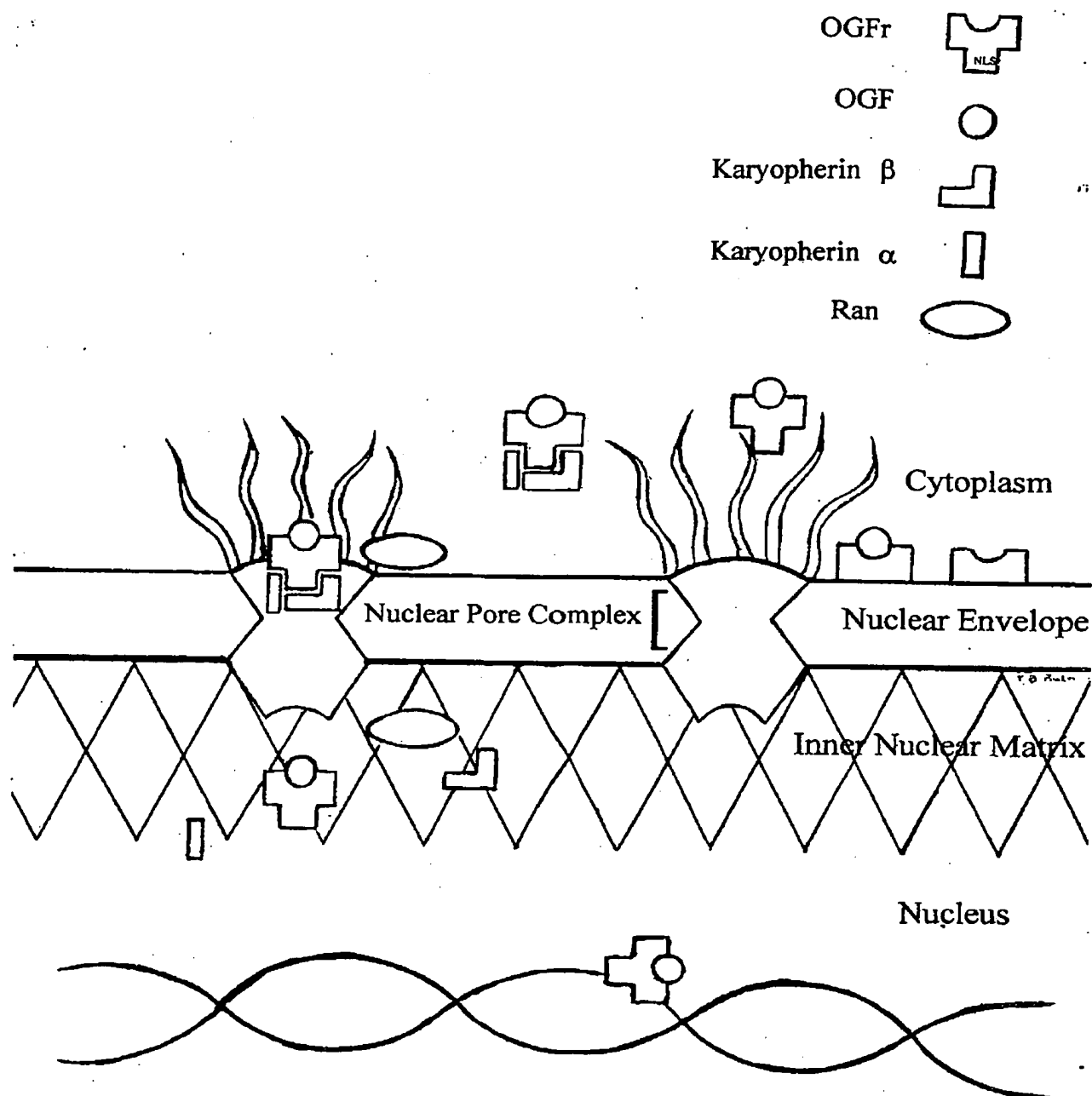


Fig. 22. Model of the relationship of the OGF receptor to interaction with OGF and the pathway for transduction of alterations in DNA synthesis.

gene expression, may play a role in the course of these cancers. Whether OGFr and OGF are directly involved with these neoplasias, or are the result of secondary consequences to other disease processes, is unclear at present. However, we could postulate from our data that diminution of the OGF receptor would allow growth to accelerate because of the inability of OGF to have its

signal transduced by the low abundance of receptors, thereby exacerbating carcinogenesis. Therefore, future studies need to be focused on examination of the entire pathway of the production, function, and degradation of the OGF receptor.

Finally, with knowledge about the cell and molecular biology of the OGF receptor, studies about the structure

and function of this receptor—and its interaction with OGF—can be undertaken. With respect to the signal transduction system for OGF, investigation of the NLS and the pathway to association with the changes in DNA synthesis are required. The domains of the OGF receptor, including domains for binding and function, need to be defined. Perhaps combinatorial libraries will be useful in the design of more specific ligands—both agonists and antagonists for the OGF receptor. It is also feasible to begin to consider using the technique of homologous recombination to 'knock out' the gene encoding the OGF receptor, and to utilize transgenic animals for other permutations to delineate the OGF receptor. Exciting times lie ahead for elucidating the relationship of OGF and OGF to the biology of growth.

Acknowledgements

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